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CHARACTERIZATION OF AND MYC EXPRESSION IN A HL-60 MUTANT RESISTANT TO DMSO INDUCED DIFFERENTIATION

Walter Michael Stadler

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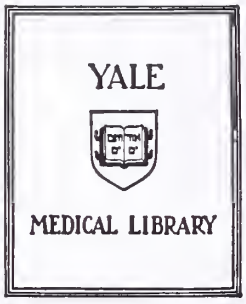
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CHARACTERIZATION OF AND *MYC* EXPRESSION IN A HL-60 MUTANT RESISTANT TO DMSO INDUCED DIFFERENTIATION

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of
Doctor of Medicine

by

Walter Michael Stadler

1988

ABSTRACT

CHARACTERIZATION OF AND MYC EXPRESSION IN A HL-60 MUTANT RESISTANT TO DMSO INDUCED DIFFERENTIATION

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The promyelocytic cell line, HL-60, has been extensively studied for its ability to terminally differentiate in response to various chemical inducers, including, but not limited to, DMSO and retinoic acid. It has also been noted that the myc oncogene is amplified and highly expressed in these cells. Furthermore, differentiation in this line, as well as in several other cell lines, is accompanied by a marked decline in myc expression, leading to the hypothesis that this decline is mechanistically important for differentiation to occur. To probe this phenomenon more closely and to test this hypothesis I isolated an HL-60 mutant that no longer responded to DMSO induced differentiation and measured myc expression in these cells following exposure to DMSO, retinoic acid, or a combination of the two. I show that such a mutant is also partially cross resistant to retinoic acid induced differentiation, that the myc gene locus is not rearranged or changed in amplification status, and that a decline in myc expression can be associated with an undifferentiated, replicating phenotype.

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AKNOWLEDGEMENTS

Like most large pieces of work, this was not completed in a vacuum. Many people were actively involved in helping, advising, and encouraging me. Foremost among these must be my friends and family who not only shared in my joy but who also put up with my complaints. Dr. David Frank deserves special mention for isolating the original mutant and for many of the ideas developed here. Dr. Ivan King was instrumental in teaching me most of the techniques used as well as serving as a critical guide. Peter Ho has my thanks for acting as an initial sounding board for many of my ideas. In addition, there were many individuals within the lab who were a great source of help. Although there are too many to mention by name, my thanks goes out to each and every one. Finally, this work would have been impossible without the support of my research advisor, Dr. Alan Sartorelli.

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ABBREVIATIONS

A260, A280: absorbence of a solution at 260 or 280 nm, respectively

DMSO : dimethyl sulfoxide

HMBA : N,N-hexamethylene bisacetamide

MOPS : buffer consisting of 20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate and 1 mM EDTA (pH 7.0)

NBT : nitroblue tetrazolium

NP40 : octylphenol ethylene oxide condensate, a nonionic detergent

PBS : phosphate buffered saline

RA : all trans retinoic acid

SDS : sodium dodecyl sulfate, an ionic detergent

SSC : buffer consisting of 150 mM NaCl, 15 mM sodium citrate (pH 7.0)

TE : buffer consisting of 10 mM Tris-HCl, 1 mM EDTA Number following is pH

TPA : 12-O-tetradecanoylphorbol-13-acetate

INTRODUCTION

The HL-60 cell line was originally isolated from a patient with acute promyelocytic leukemia (1,2). It has been shown that a variety of agents can induce these cells to differentiate along either the granulocytic, monocytic, or even eosinophilic pathways (3-15). The morphological maturation is accompanied by numerous functional and cytochemical changes, including the acquisition of the ability to reduce NBT, an assay for the ability of phagocytic cells to generate an oxidative burst (4). Although most of these changes approximate the characteristics of normal granulocytes or macrophages, it has been pointed out that there are significant differences (16,17). Of particular note is the ability of uninduced HL-60 cells to divide continuously in culture, an unusual characteristic even of most leukemias, and their grossly abnormal karyotype (2), which does not correspond to any commonly recognized association between karyotype and malignancy (18).

Yet, like normal terminally differentiated cells, induced HL-60 cells have a finite lifetime and cannot be maintained in culture or cloned (19). It is for this reason, that they are believed to be a good model system to investigate differentiation mechanisms and to explore differentiation induction as a possible therapeutic modality.

Oncogenes were first isolated from transforming retroviruses (20) and subsequently from NIH3T3 cells transfected

with tumor DNA (21). They have since been identified in normal cells and their elevated expression or mutated forms identified in numerous human and animal malignancies (22-26). Oncogenes are thought to be involved in regulating growth and replication. In fact, several have been shown to be growth factors or growth factor receptors (27-32). The protein product of others, such as the myc oncogene, have also been shown to reside in the nucleus and to bind double stranded DNA (33). Since a cardinal manifestation of a malignant cell is an undifferentiated phenotype, a role for some oncogenes in maintaining such a state or in initiating the differentiated state was soon hypothesized and explored. One of the first such investigations used the HL-60 leukemia and showed that during differentiation the RNA expression of the myc oncogene was greatly reduced (34). It had already been shown that the myc gene locus is amplified in these cells (35,36). Subsequent work showed changes in other oncogenes, including a similar decline in the expression of another nuclear oncogene, myb (37), and extended these observations to other differentiating cell lines (38-40).

In further studies, the kinetics of the decline in myc expression were more carefully defined (19,41), demonstrating that they are mostly due to a specific decline in the myc transcription rate (42). The decrease in expression is also accompanied by a slight change in S1 nuclease sensitive sites of the myc gene (43). Finally, constitutive expression of

myc in a mouse erythroleukemia line inhibited DMSO or HMBA induced differentiation (44-46), but transgenic mice with a similarily activated myc locus developed normally (47,48). Thus, although it seemed that high myc levels were sufficient to maintain the undifferentiated replicating phenotype, there was no direct information as to whether high levels of expression were necessary.

To analyze the importance of myc expression to the differentiation process, I developed a mutant HL-60 line which no longer responded to DMSO induced differentiation and measured the effects of DMSO exposure on myc expression. Several other investigators have reported isolating DMSO resistant HL-60 cells (49-52). One report documented cross-resistance to several other inducers, as well as a reduction in the myc gene copy number (51). Accompaning changes in the expression of several cell membrane proteins were also noted, but myc RNA expression was not measured (53). Another report showed a slight decline in myc expression in a DMSO resistant mutant, but the decrease was only 2-fold and only dot-blots were used to document the finding (52). Here I report a marked decline in myc RNA expression following DMSO exposure in mutant HL-60 cells unable to respond to DMSO induced differentiation.

METHODS

CELL CULTURE

HL-60 cells were originally obtained from Dr. Robert C. Gallo of the National Cancer Institute (1) and stock cultures were maintained and stored at our facilities. Cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 ug streptomycin at 37°C in a 5% CO_2 humidified incubator. They were kept in exponential phase by splitting every 2-3 days and were periodically tested to insure freedom from mycoplasma infection. Every set of experiments was conducted by growing a sufficient number of cells such that all treatments and time points were begun concurrently and with the same set of seed cultures. All media were prewarmed to 37°C.

The mutant cells were isolated as described in the Results section and were maintained in the manner described above except that the medium was also supplemented with 1.2% DMSO. Before beginning an experiment, cells were washed in drug-free medium and passaged once in drug-free medium before resuspending in the appropriate treatment flasks. All cell counts were obtained on a Coulter model ZBI particle counter.

NBT TEST AND STAINING

To measure differentiation, cells were cytospun onto acid-washed slides, fixed, and stained for morphological

evaluation by a rapid staining solution (Canko Quik Stain II) that approximates the Wright-Giemsa stain. The NBT test was conducted as previously described (4). One x 10^6 cells were resuspended in 0.5 ml of medium and 0.5 ml of 0.2% NBT (made up in sterile PBS protected from light, and stored at 5^oC). TPA (from a 1 mM stock solution made up in DMSO, protected from light, and stored at -20^oC) was added to a final concentration of 0.1 uM and the suspension incubated at 37^oC for 20 min. The reaction was stopped by placing the solution on ice, the cells were collected by centrifugation, resuspended in a minimal amount of medium, and the percentage of cells containing black formazan particles was measured using a standard counting chamber. All cell counts (for both morphology and NBT reduction) were done blindly, in at least duplicate, and at least 200 cells were counted each time.

NORTHERN ANALYSIS

RNA was isolated by the guanidine thiocyanate method (54) and analysed by Northern blotting (55,56). Briefly, cells collected by centrifugation were washed in ice-cold PBS, and resuspended in 0.7 ml of 4 M guanidine thiocyanate in 25 mM sodium citrate with 0.1 M 2-mercaptoethanol (pH = 7.0). Cells were immediately vortexed and 0.5% Sarkosyl was added to complete the cellular disruption. This mixture was layered over 4 ml of 5.7 M CsCl in 25 mM sodium citrate (pH = 5.0) and centrifuged at 32K RPM in a Beckman

SW-40 rotor for 18 h at 20°C. The RNA pellet was resuspended in sterile water and reprecipitated. The final RNA pellet was resuspended in TE7.5 and quantitated spectrophotometrically by A260.

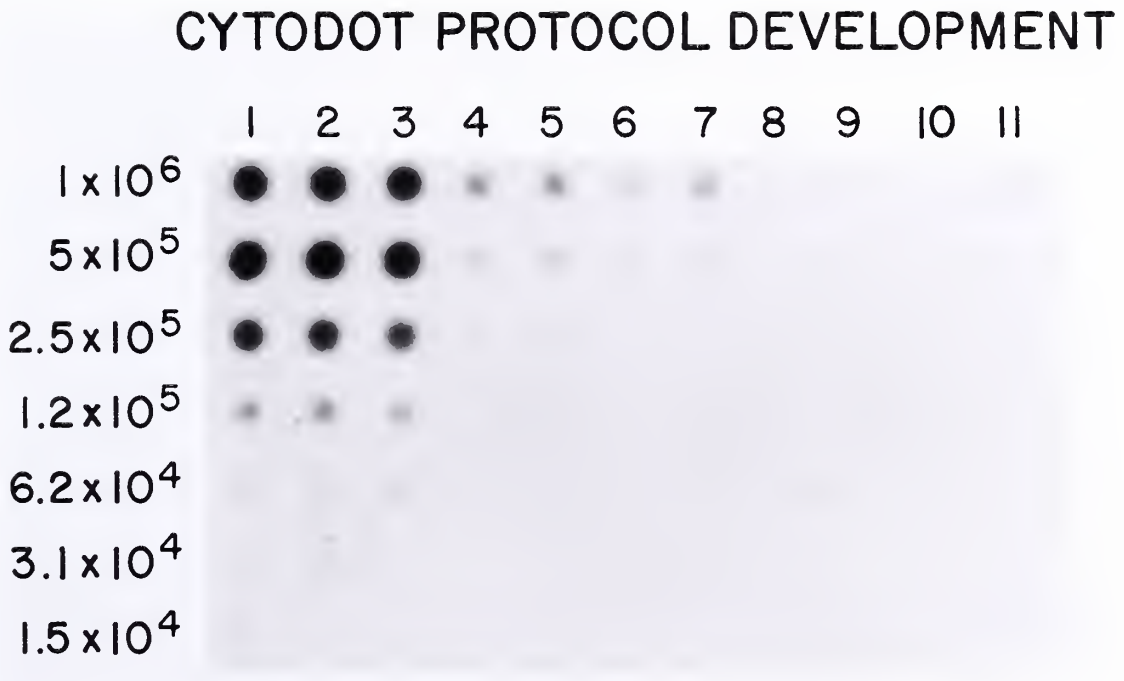
Ten ug of RNA was heat-denatured in 50% formamide and 6% formaldehyde and loaded onto a 1.2% agarose formaldehyde gel. After electrophoresis in 1x MOPS, the 18S and 28S bands were visualized by acridine orange staining and the RNA transferred to nitrocellulose with 5x SSC.

The baked filters were prehybridized in a buffer containing 50% formamide and 10% dextran sulfate at 42°C for 2 to 12 hr and then hybridized in the same solution for approximately 24 hr at 42°C. Blots were rinsed in 2x SSC with 0.5% SDS at room temperature, washed for 15 min in 2x SSC with 0.5% SDS at room temperature, washed for 20 min in 1x SSC with 0.1% SDS at 42°C, and finally washed for 30 min in 0.5x SSC with 0.1% SDS at 65°C. The blots were exposed to Kodak XRP film for 24 to 48 hours at -70°C.

CYTODOT ANALYSIS

Cytodot analysis was accomplished by a modification of a published technique (57) that was arrived at after systematic analysis (see Figure 1). Two x 10^6 cells were washed twice in ice-cold PBS and resuspended in 45 ul of TE7.0 and 1000 units of RNAsin. Five ul of 5% NP40 were added and the mixture incubated on ice for 5 min. Another 5 ul of 5% NP40

FIGURE 1: Cytodot probed with v-myc. Lane 2 cells treated with NP40, RNasin, and Centrifugation as described in Methods, other lanes had the following changes. 1,10: no RNAse inhibitors added; 3,5,7,9: vanadyl nucleoside analogs replace RNAsin; 4-7,10: phenol/chloroform extraction of supernatant added; 6-10: proteinase K treatment of supernatant added; 11: same as 6, but RNAse added at end.


CYTODOT PROTOCOL DEVELOPMENT
1 2 3 4 5 6 7 8 9 10 11
1 x 10^6
5 x 10^5
2.5 x 10^5
1.2 x 10^5
6.2 x 10^4
3.1 x 10^4
1.5 x 10^4

were added and the mixture was incubated on ice for a further 5 minutes. The nuclei were collected by centrifugation and 50 ul of the resulting suspension were added to 30 ul of 20x SSC. The RNA was denatured with 7% formaldahyde at 60°C for 20 min and the amount of solution representing 1 x 10^6 cells was placed on Gene-Screen with a Minifold blotting apparatus. Dilutions (1:2) were made with 15x SSC. The filters were baked and blotted as above.

SOUTHERN ANALYSIS

DNA was isolated from cells by a phenol/chloroform method and analyzed by Southern blotting (56,58). Briefly, cells were collected by centrifugation, washed in ice-cold PBS, resuspended in TE8.0, and lysed with 10 volumes of a 0.5 M EDTA, 0.5% Sarkosyl, and 100 ug/ul proteinase K solution at 50°C. The nucleic acids were extracted by repeated phenol, phenol/chloroform, and chloroform extractions and the solution dialyzed against TE8.0. RNA was removed by treating the solution with DNAse-free RNAse followed by repeated phenol/chloroform extractions. After a second dialysis against TE8.0, the DNA was concentrated by reprecipitation, and quantitated spectrophotometrically by A260. Agarose gel electrophoresis showed that all of the DNA so isolated was greater than 23kb.

Approximately 12 ug of DNA were cut by restriction enzymes (56), the fragments separated on a 1.2% agarose gel,

and then transferred to nitrocellulose. Prehybridization, hybridization, and washing were as for the Northern analysis.

PROBES

For Southern blots, a v-*myc* probe (59) provided by Dr. Mark Carmen was used. For Northern blots, a murine c-*myc* probe (60,61) and a human N-*ras* probe isolated from HL-60 cells (62), both of which were received from the American Type Culture Collection, were used. Probes were isolated from *E. coli* using standard procedures (56).

All probes were labeled to approximately 9×10^8 cpm/ug by an oligonucleotide labeling technique (63). Briefly, denatured probe was incubated with oligonucleotides, dATP, dGTP, dTTP, ^{32}P-dCTP, and the Klenow fragment of bacterial DNA synthetase. Free nucleotides were separated from the labeled probe by passage over a G-50 column.

DENSITOMETRY

Radioautographs were densitometrically scanned using a Zeineh soft laser scanning densitometer equipped with an Apple computer which was used for data analyses.

CHEMICALS AND SOLUTIONS

All chemicals used were of the highest grade available and supplied by standard laboratory suppliers. All solutions were sterilized in an autoclave before use. In addition,

glassware for RNA work was baked at 250°C for 4 hr prior to use.

RESULTS

CELL ISOLATION AND CHARACTERIZATION

Under the conditions employed parental HL-60 cells had a doubling time of about 36 hours and plateaued after 3 doublings. When treated with 1.2% DMSO or 1 uM RA, their growth was inhibited and they acquired a differentiated phenotype as assayed by the ability to reduce NBT (Figure 2).

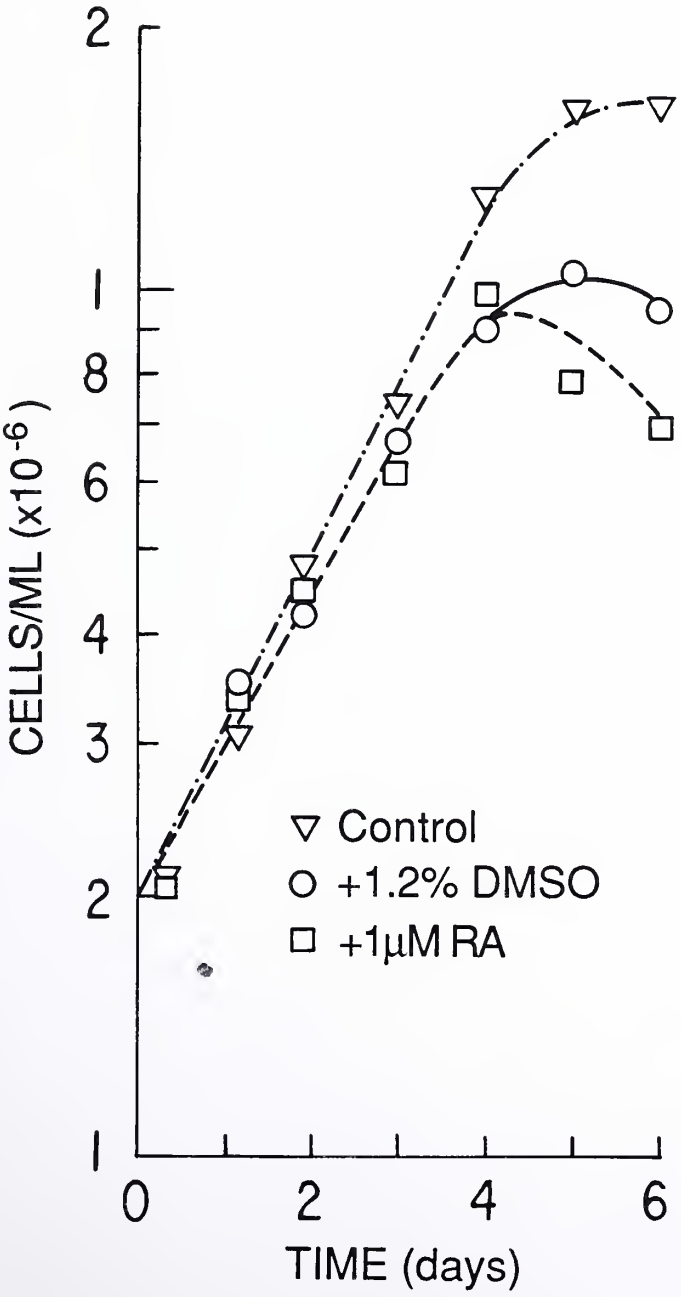
To create an HL-60 line resistant to DMSO induced differentiation, parental HL-60 cells were continuously maintained in medium with serially increasing concentrations of DMSO until a culture resistant to 1.2% DMSO was created. This was successful in three instances. In each case the cells grew well in 1.2% DMSO (albeit somewhat more slowly than the parental line) without showing any ability to reduce NBT. However, these resistant lines died when placed in drug-free medium, indicating a dependency on DMSO (*). Because of the close resemblance to the growth characteristics of the parental line, one of these resistant lines, termed HL-60-DA, was selected for further study.

Figure 3 shows the growth and differentiation of HL-60-DA cells in DMSO concentrations ranging from 1.2% to 2.2%. The cells grew well in 1.2% to 1.6% DMSO; however, they demonstrated up to 20% NBT positivity at this latter concentration. As the DMSO was further increased, the cells grew less well and demonstrated greater NBT positivity. A

*This initial work was carried out by David Frank, MD, PhD.

FIGURE 2: HL-60 growth and differentiation (inset). Conditions and measurments are as described in Methods. Each point represents a separate flask and is the average of at least duplicate samples.

HL-60 GROWTH AND DIFFERENTIATION



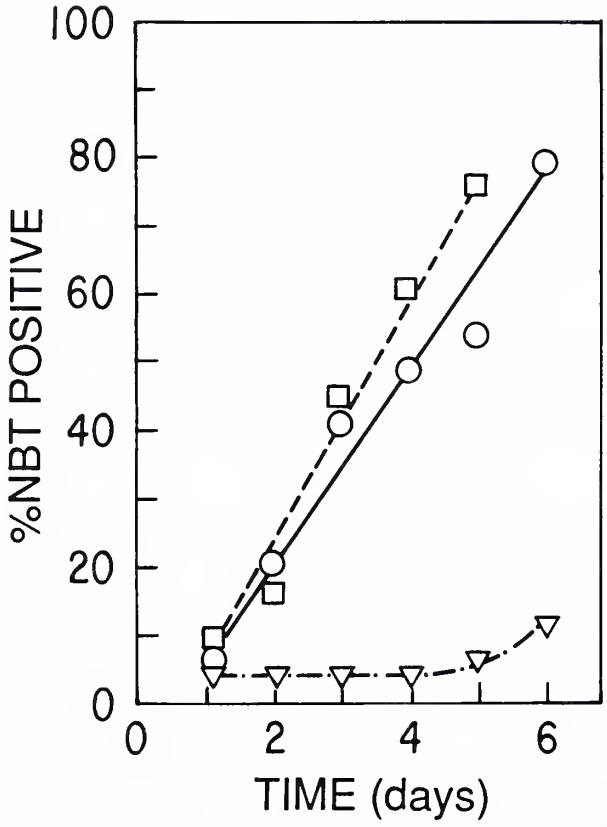
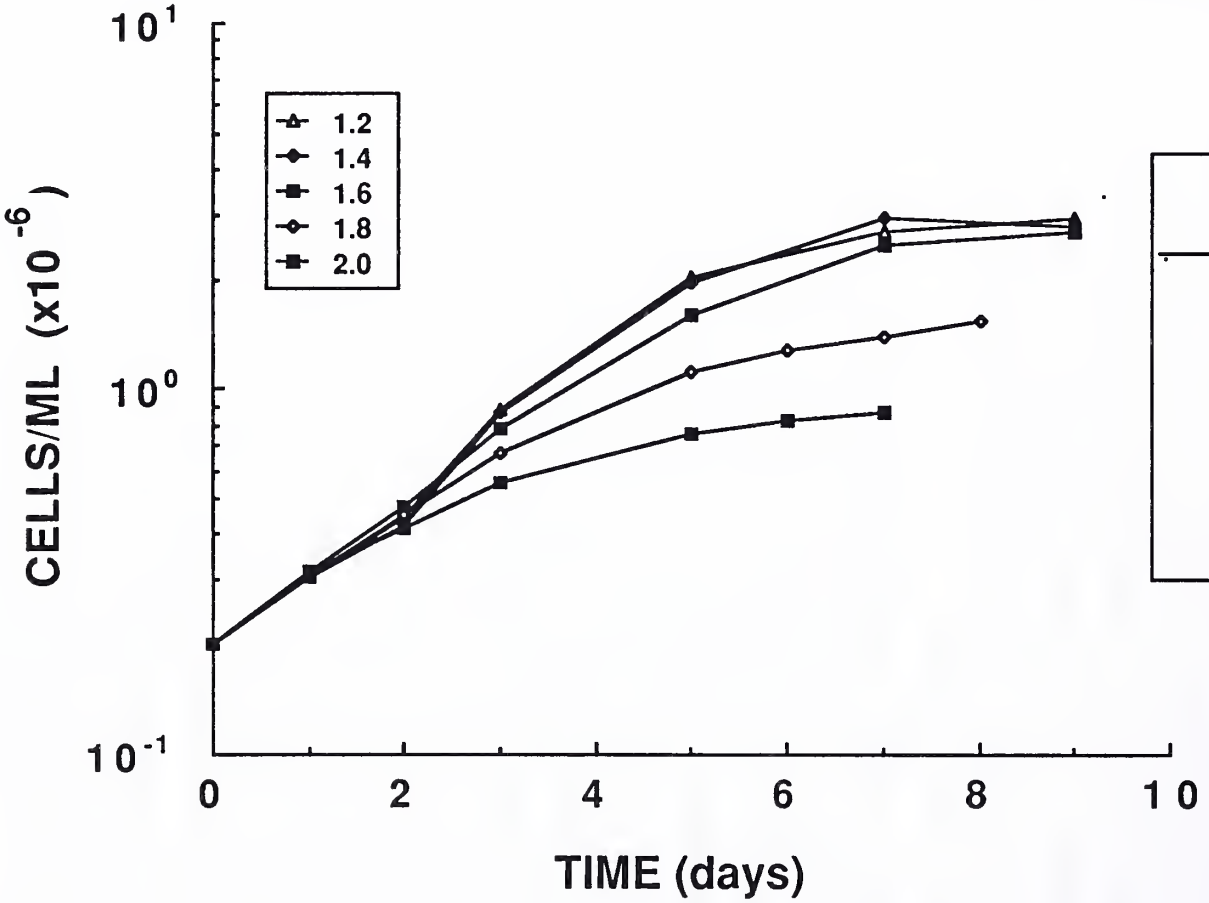


FIGURE 3: HL-60-DA dose response to DMSO. Culture conditions and measurments are as described in Methods. The accompaning tabular insert shows the percentage of cells capable of reducing NBT on the last day of culture. Cells treated with 2.2% DMSO were not assayed due to the large amount of cellular debris.

HL-60-DA DOSE RESPONSE



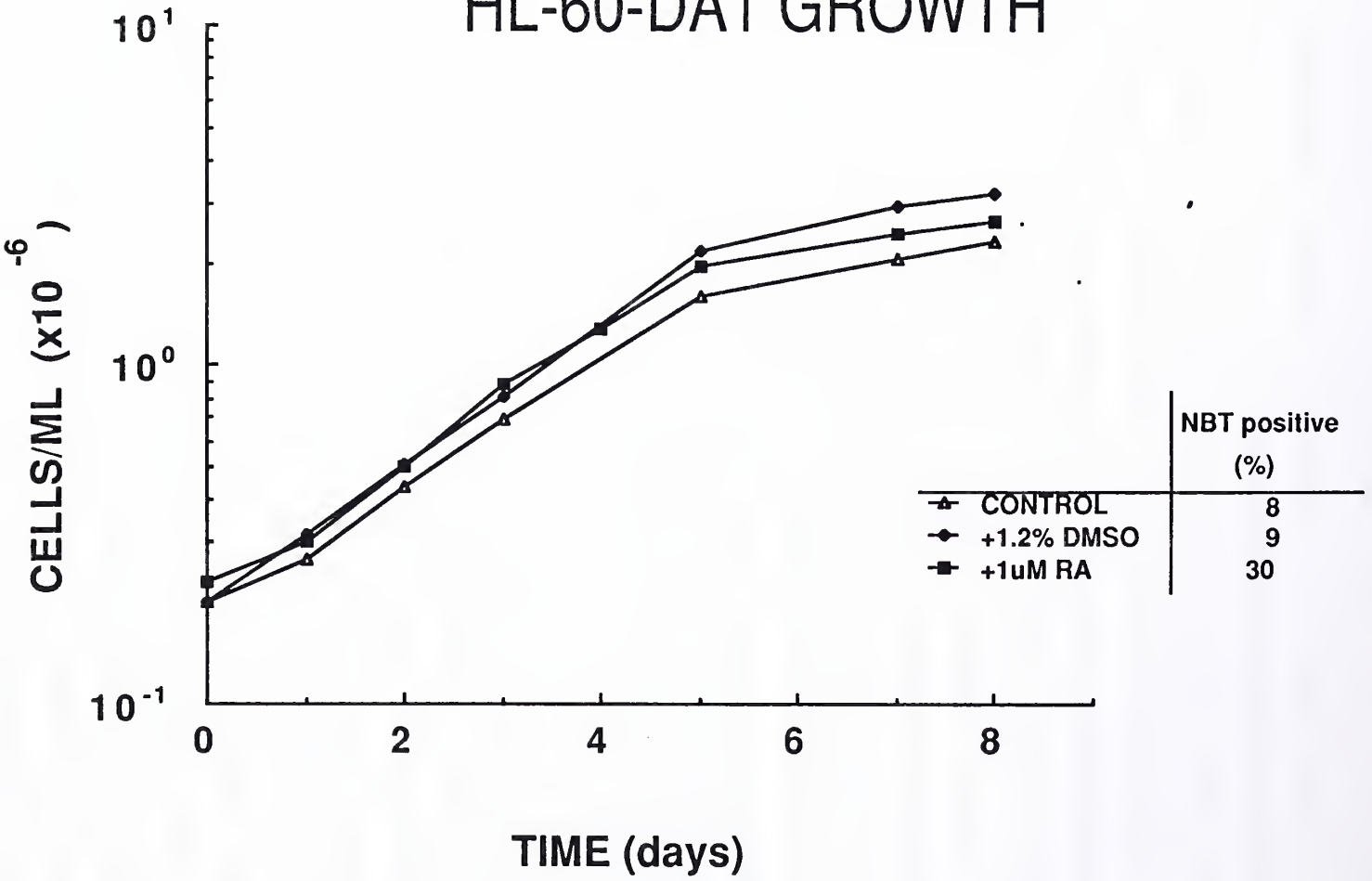
DMSO (%)	NBT positive (%)
1.2	4
1.4	6
1.6	20
1.8	52
2.0	48
2.2	-

DMSO concentration of 2.2% was toxic, as demonstrated by cellular vacuolation and debris after 3 days in culture.

As mentioned above, it has been reported that myc expression decreases dramatically in parental HL-60 cells upon DMSO induced differentiation (34). Therefore, I assayed myc RNA levels in HL-60-DA cells during exponential growth, as well as during plateau phase. An RNA "dot blot" showed that myc expression in the mutant line was significantly lower than in the uninduced parental line and was approximately as low as in DMSO induced parental HL-60 cells (data not shown). These studies, however, were complicated by the necessity of continuously maintaining the mutant cell line in DMSO. It was impossible, therefore, to determine whether this decrease was a DMSO-induced effect or a permanent alteration. For this reason, I attempted to isolate a cell line that remained resistant to DMSO induced differentiation but was not dependent on it for growth.

To this end, some HL-60-DA cells were placed in drug-free medium. In contrast to earlier results, after two passages a cell line developed that grew well both in the presence and absence of DMSO. This line, now termed HL-60-DA1, was maintained in drug-free medium. Figure 4 shows the growth and NBT positivity of HL-60-DA1 cells in 1.2% DMSO, in 1 uM RA, and in drug free medium. This cell line grew slightly better in the medium with DMSO than in the drug-free medium, but failed to differentiate in either case. Surpri-

FIGURE 4: Growth of HL-60-DA1 cells in presence of DMSO or RA Culture conditions and measurements are as described in Methods. The accompaning tabular insert shows the percentage of cells capable of reducing NBT on the last day of culture.


HL-60-DA1 GROWTH
CELLS/ML (x10^-6)
10^1
10^0
10^-1
0
2
4
6
8
TIME (days)
NBT positive (%)
CONTROL 8
+1.2% DMSO 9
+1uM RA 30

singly, the cells also grew well, and differentiated only partially (30% NBT positivity) when exposed to RA. Control HL-60 cells cultured at the same time and induced with either RA or DMSO showed the expected growth inhibition and greater than 75% NBT positivity at plateaued growth, thus demonstrating that the assays were functioning properly. Subsequent experiments demonstrated that the growth of HL-60-DA1 cells was inhibited and differentiation occurred, with the cells expressing greater than 75% NBT positivity, when they were placed in both 1.2% DMSO and 1uM RA (data not shown), thus proving that the resistance to differentiation was not absolute.

The HL-60-DA1 cells maintained their mutant phenotype for approximately fifteen passages in drug-free medium and it was initially felt that this was a stable mutant. Unfortunately, as the cells continued to be passaged in drug-free media in preparation for measuring myc RNA expression, it was noted that they began to revert back to the parental phenotype. For this reason, some of the early passage HL-60-DA1 cells were removed from frozen storage and placed in medium supplemented with 1.2% DMSO to maintain the selective pressure. Unlike the parental line, the recovery of viable cells from the frozen stock of HL-60-DA1 cells was extremely low and estimated to be less than 1%.

A viable culture was eventually created and these cells, now termed HL-60-DA2, were maintained in 1.2% DMSO until

ready for study. Figure 5 shows their initial growth and differentiation. Although qualitatively similar to the original HL-60-DA1 cells, they do show some differences. They still grow well in drug free, DMSO treated, or RA treated media, but now show up to 50% NBT positivity with RA treatment. In addition, cells treated with both RA and DMSO also developed only 50% NBT positivity, although they did so more rapidly than those treated with RA alone. A control HL-60 culture grown and assayed at the same time once again responded as usual. To further characterize these cells, Wright-Giemsa stained samples from the cultures were examined for morphological changes (Figure 6). The cells in drug-free or DMSO treated media both matured slightly from predominantly promyelocytes to approximately 50% myelocytes. The drug-free cells seemed to mature somewhat more extensively, but this was not statistically significant ($p > 0.05$ by X^2 analysis). Cells treated with both RA and DMSO differentiated so that a majority of the cells resembled metamyelocytes or bands. Unfortunately, the sample for the 8-day treatment with RA was lost thereby making analyses difficult. These findings confirmed the NBT results and correlated well with them, in so much as metamyelocytes are considered the earliest cells capable of expressing NBT positivity.

Stock HL-60-DA2 cells were continuously maintained in 1.2% DMSO for two months, at which time the confirmation of the above results, as well as the results of the myc analysis

FIGURE 5: HL-60-DA2 growth and differentiation. Culture conditions and measurements are as described in Methods.

HL-60-DA2 GROWTH AND DIFFERENTIATION

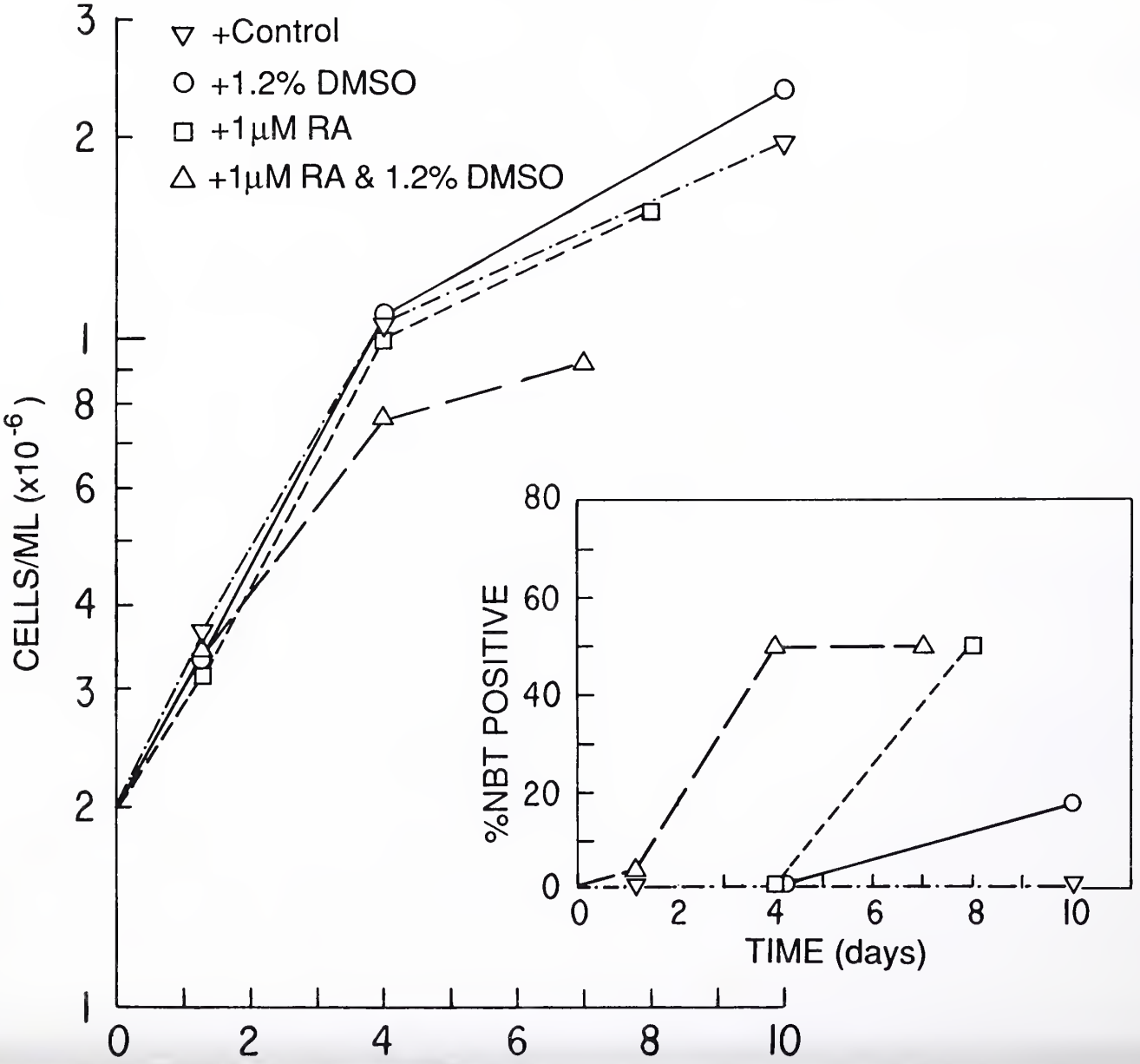
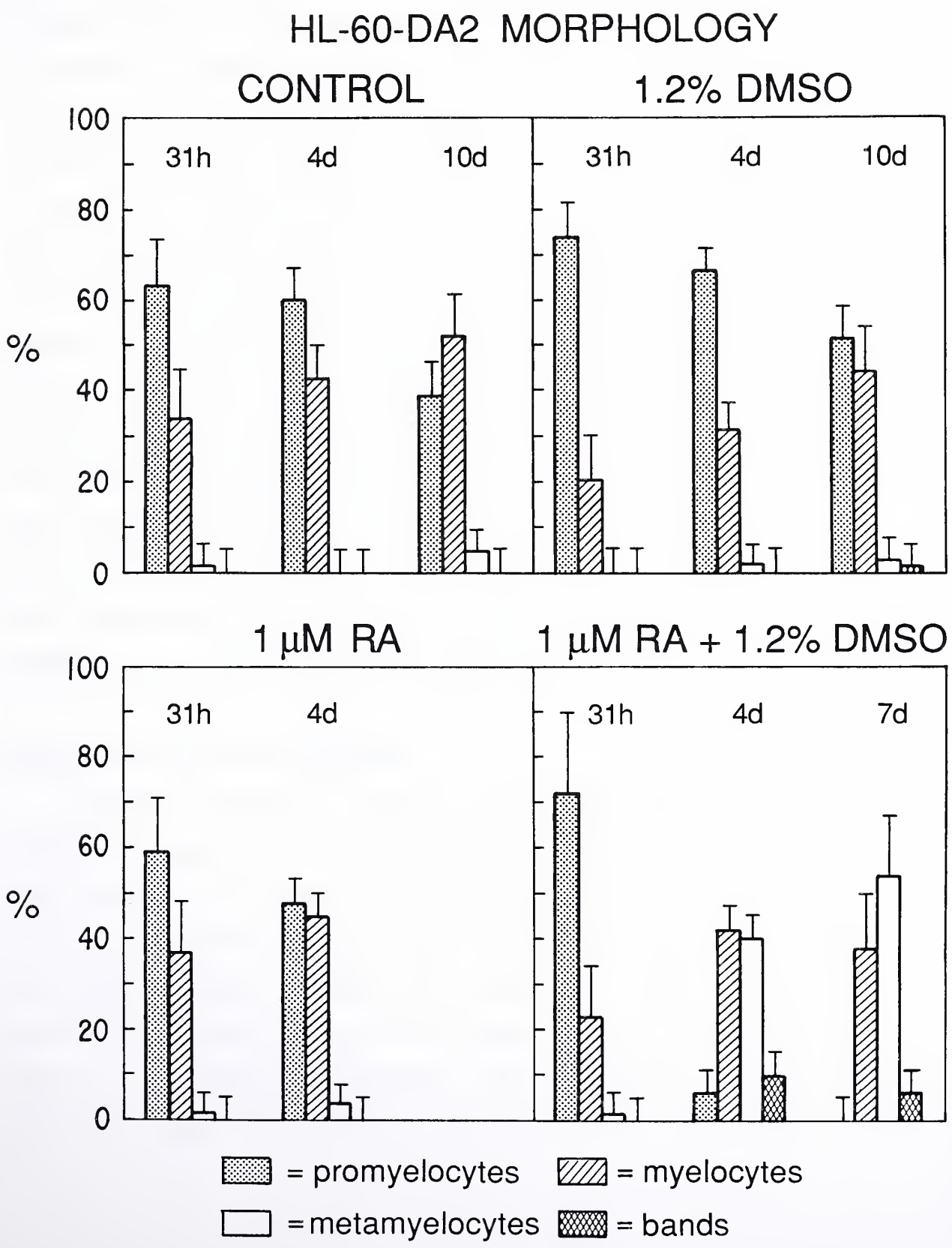


FIGURE 6: Morphological differentiation of HL-60-DA2 cells. Assay performed as described in Methods. All samples were blinded, at least 200 cells were counted, and the results represent the average of at least duplicate measurements. Error bars represent ± one standard deviation or 5%, whichever is greater.


HL-60-DA2 MORPHOLOGY
CONTROL
1.2% DMSO
1 μM RA
1 μM RA + 1.2% DMSO
%
100
80
60
40
20
0
31h
4d
10d
7d
= promyelocytes
= myelocytes
= metamyelocytes
= bands

discussed below, was attempted. The experiment was repeated in the exact manner as before except that the cells were cultured in smaller flasks. Figure 7 shows their growth and NBT positivity and Figure 8 the morphological analysis. It is clear that the cellular characteristics had changed and thus they are now termed HL-60-DA3 cells. These cells grew well even in the presence of both RA and DMSO and expressed a maximum of 10% NBT positivity under those conditions. Morphology confirmed these results for the drug-free, the DMSO treated, and the RA treated cultures. The cultures treated with both RA and DMSO developed more metamyelocytes and bands than would be predicted from the NBT test but still not as many as the HL-60-DA2 cells (see Figure 6). Parental HL-60 cells cultured and tested at the same time and with the same reagents once again behaved as expected.

ANALYSIS OF THE MYC ONCOGENE

RNA was isolated at various times from the cells described in Figures 5 and 6 and probed for the *myc* oncogene. Since it has been shown that N-*ras* RNA levels do not change with HL-60 differentiation (19), the blots were simultaneously probed for this oncogene as a control. Figure 9 shows the Northern blots and Figure 10 the densitometric scanning results. All bands are consistent with previously reported sizes for these RNA species (34,62,64). With a few minor

FIGURE 7: HL-60-DA3 growth and differentiation. Culture conditions and measurements were as described in Methods.

HL-60-DA3 GROWTH AND DIFFERENTIATION

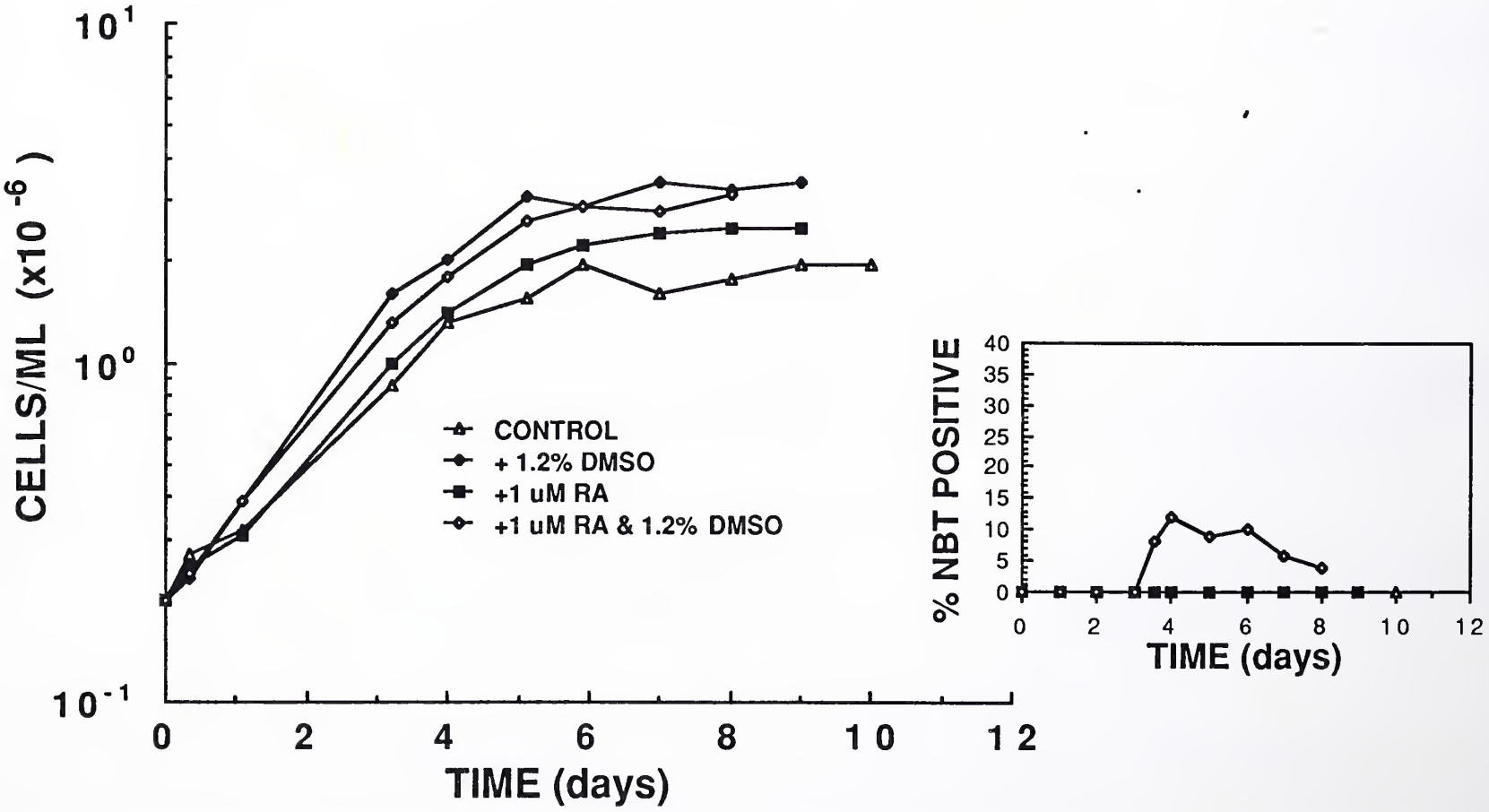


FIGURE 8: Morphological differentiation of HL-60-DA3 cells. Analyses are as in Figure 6.

HL-60-DA3 MORPHOLOGY

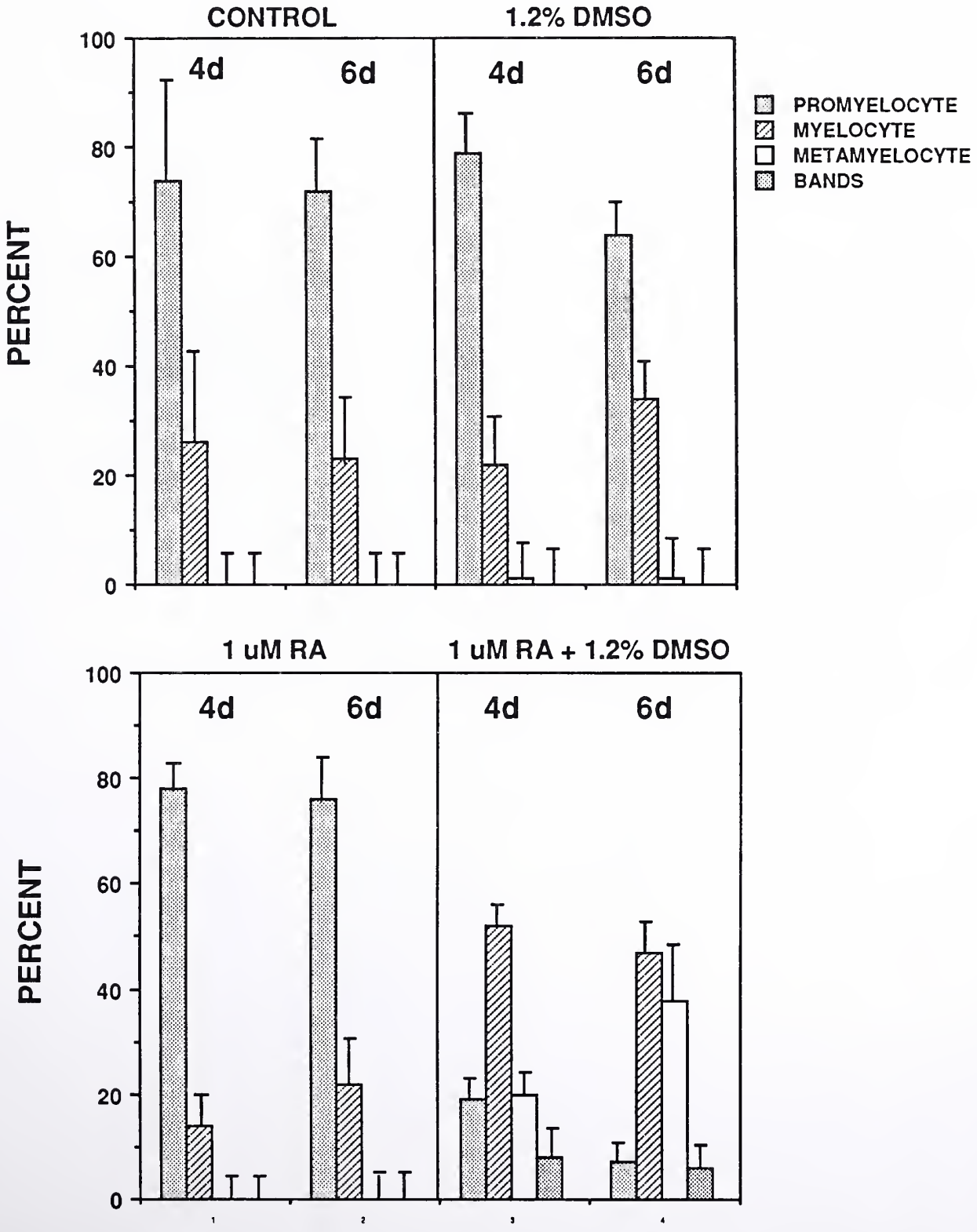
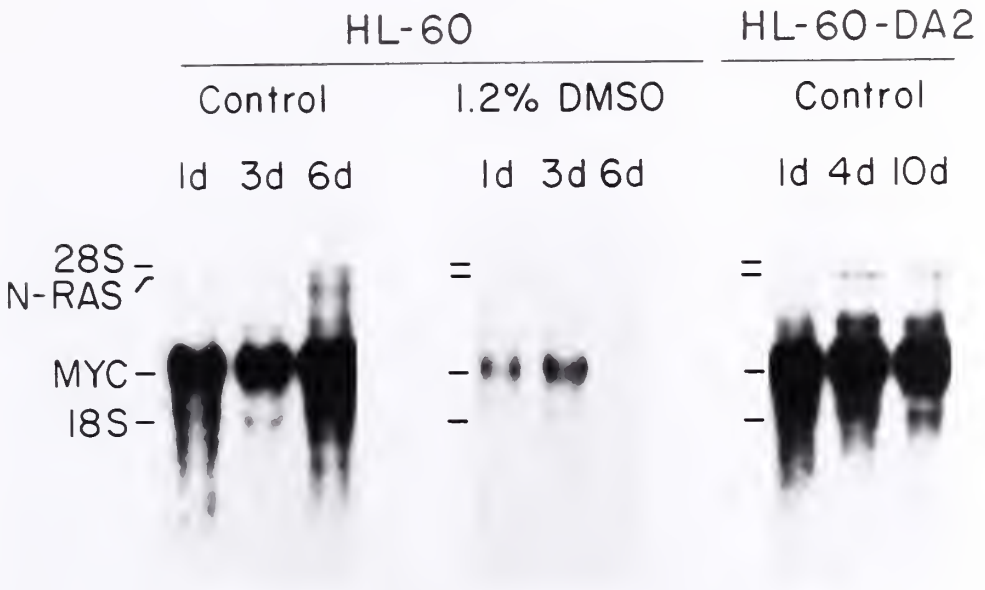


FIGURE 9: Northern analysis of total RNA isolated from HL-60-DA2 cells and probed with human N-ras and mouse c-myc. Twenty micrograms of total RNA were isolated, separated, and hybridized as described in Methods. Bands were labeled and confirmed by independent hybridizations. N-ras bands were more clearly visualized on the original autoradiograph and on overexposure of the film.

MYC AND N-RAS RNA EXPRESSION



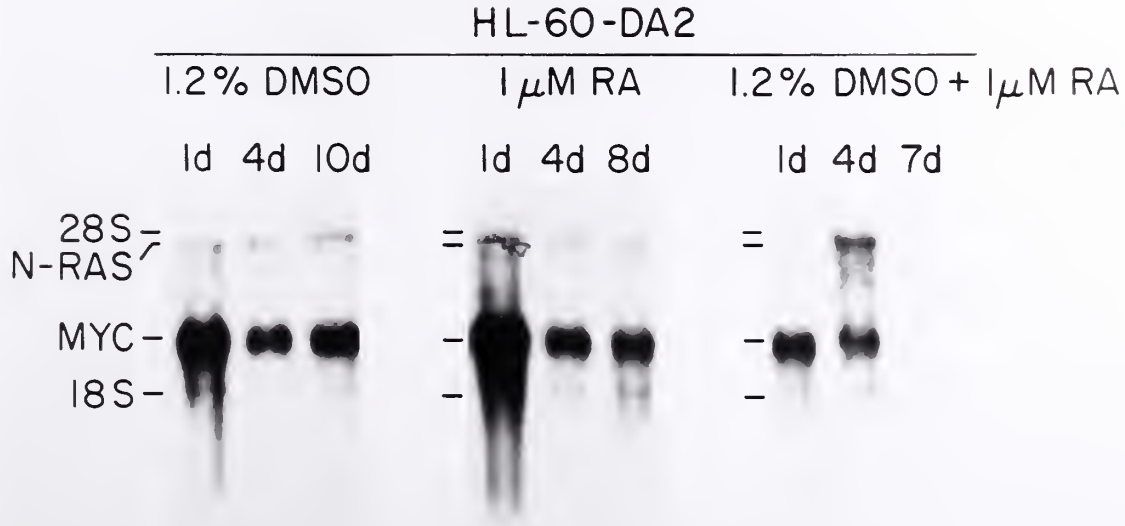
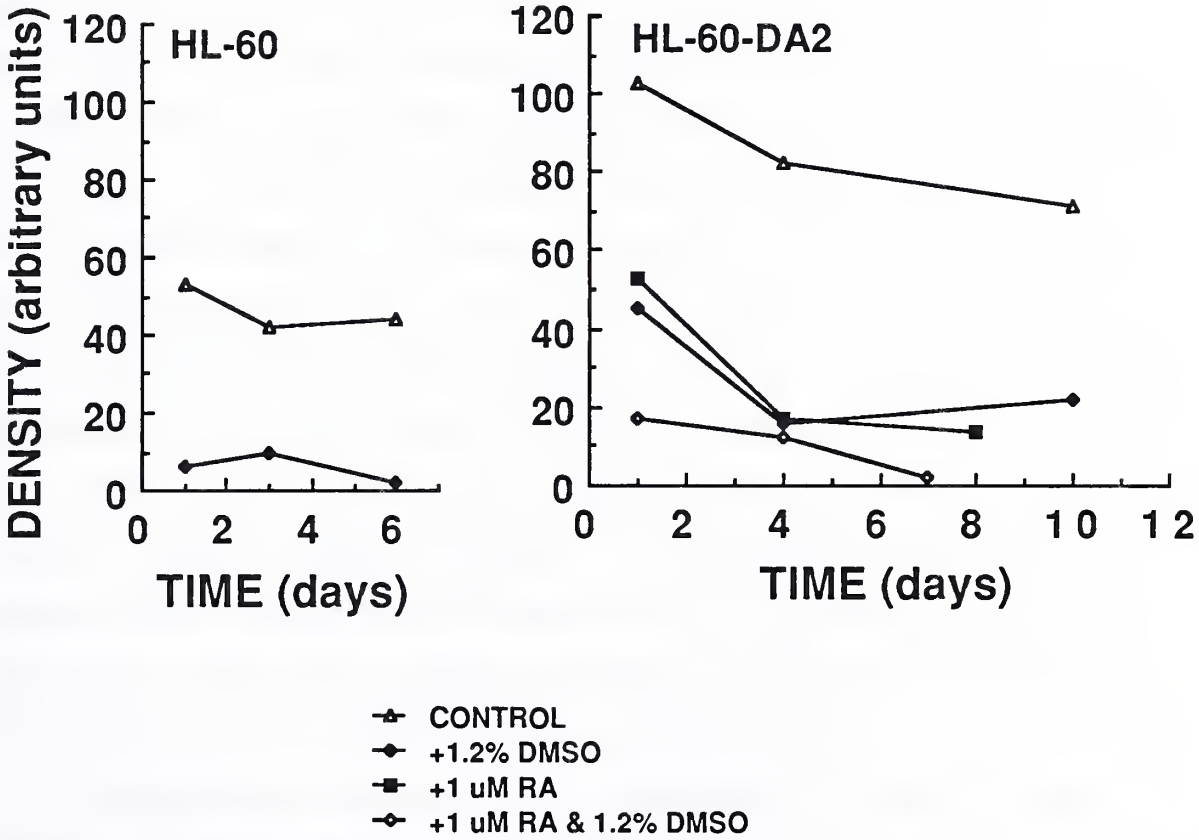


FIGURE 10: Densitometric measurements of Northern analysis from Figure 9. N-ras bands could not be scanned because of their low intensity.

MYC RNA EXPRESSION



exceptions attributable to loading variations, the RNA levels of N-ras remained relatively constant. Myc RNA levels, however, varied dramatically. First of all, the untreated parental HL-60 cells consistently expressed high levels of myc which were dramatically reduced when the cells were induced to differentiate with DMSO. The 5 to 20-fold decrease in myc expression is consistent with previously published data (19,34). The untreated HL-60-DA2 cells showed even higher levels of myc RNA expression than the untreated parental line. Given the difficulty of accurately scanning the high density bands in these experiments, it is not known if the modest decline over time seen in Figure 10 is significant. Treatment with DMSO, which in this line does not cause differentiation, once again caused a dramatic decrease in myc RNA. Treatment with RA, which causes moderate differentiation, induced similar changes. Treatment with RA and DMSO, which caused moderate differentiation at a faster rate than RA alone, produced a dramatic decline in myc RNA, similar to that seen in the differentiating parental line.

To confirm these results, I attempted to repeat them using a cytodot method (57). Preliminary experiments showed that this was a feasible technique (see Methods). This method was chosen for its rapidity and convenience in analyzing samples and time points. In addition, densitometric scanning was easier than with the Northern blots. The drawbacks include difficulty in controlling for nonspecific bin-

ding of the probe and, since RNA levels are expressed in units/cell rather than in units/ug of total RNA, difficulty in comparing the results to the earlier Northern blots. I attempted to circumvent the first by using the same stringent washing conditions as for the Northern analysis. The second difficulty, however, cannot be ameliorated, but it may be argued that the amount of myc per cell is the physiologically more important value. In addition to these drawbacks, and as described above, the characteristics of the HL-60 mutant changed in the interval between the experiments.

Despite these problems the results were very similar. Figure 11 shows some of the representative blots and Figure 12 the results from densitometric scanning. It should be first noted that the changes in myc RNA levels are not as dramatic as with the Northern blots. This finding is probably due to the inherent high levels of nonspecific binding. Nevertheless, N-ras levels did not change with treatment or over time in the parental line, while a slight decline occurred over the course of the experiment in the mutant. This decline was consistent and equal for all of the four treatments. In addition, it was observed that the density of the N-ras dots in the mutant were higher than in the parental line. It is not known if this reflects a relatively higher concentration of that RNA species within the cell or simply an artifact.

As seen with the Northern blots, myc RNA levels declined

FIGURE 11: Representative cytodots probed with human N-*ras* or mouse c-*myc*. One $x10^6$ cells were treated, blotted onto gene-screen, and hybridized as described in Methods. Dilutions and probes used are indicated.

MYC AND N-RAS RNA EXPRESSION CYTODOT ANALYSIS

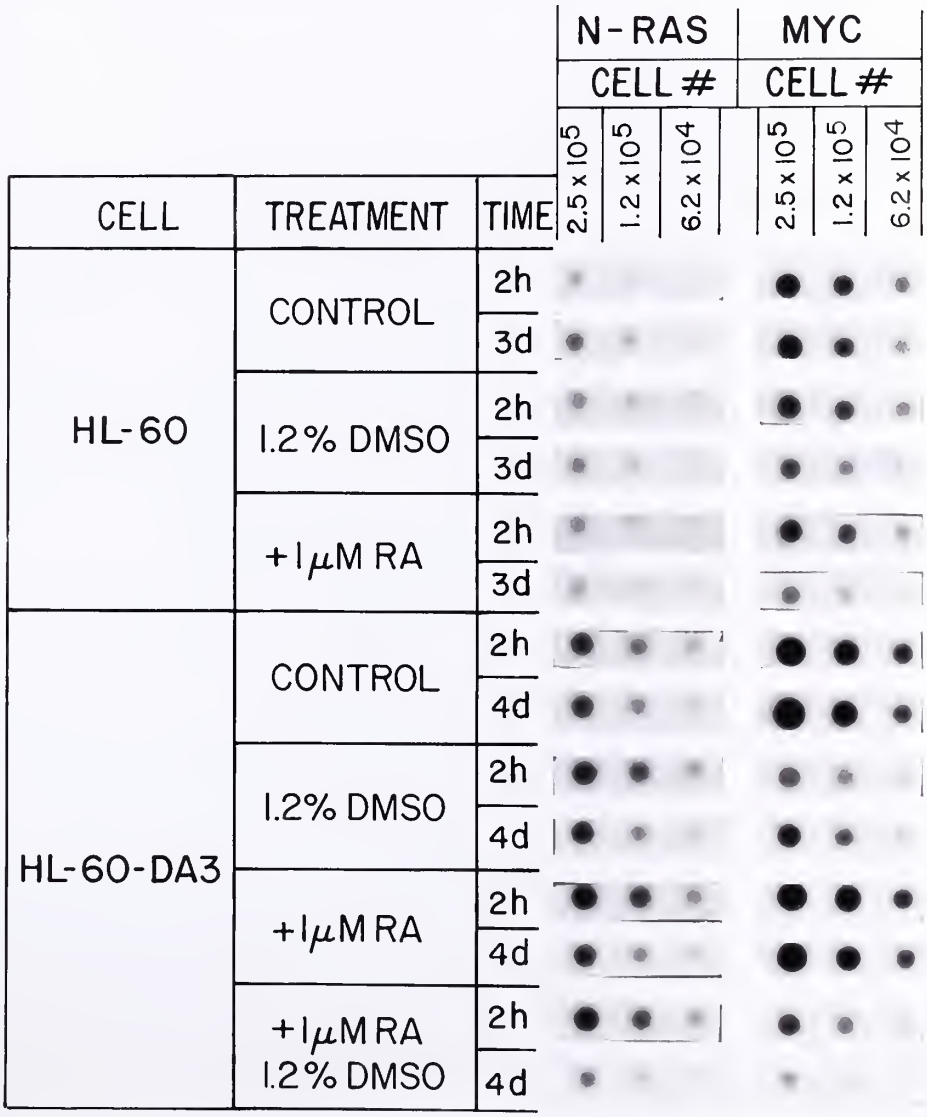
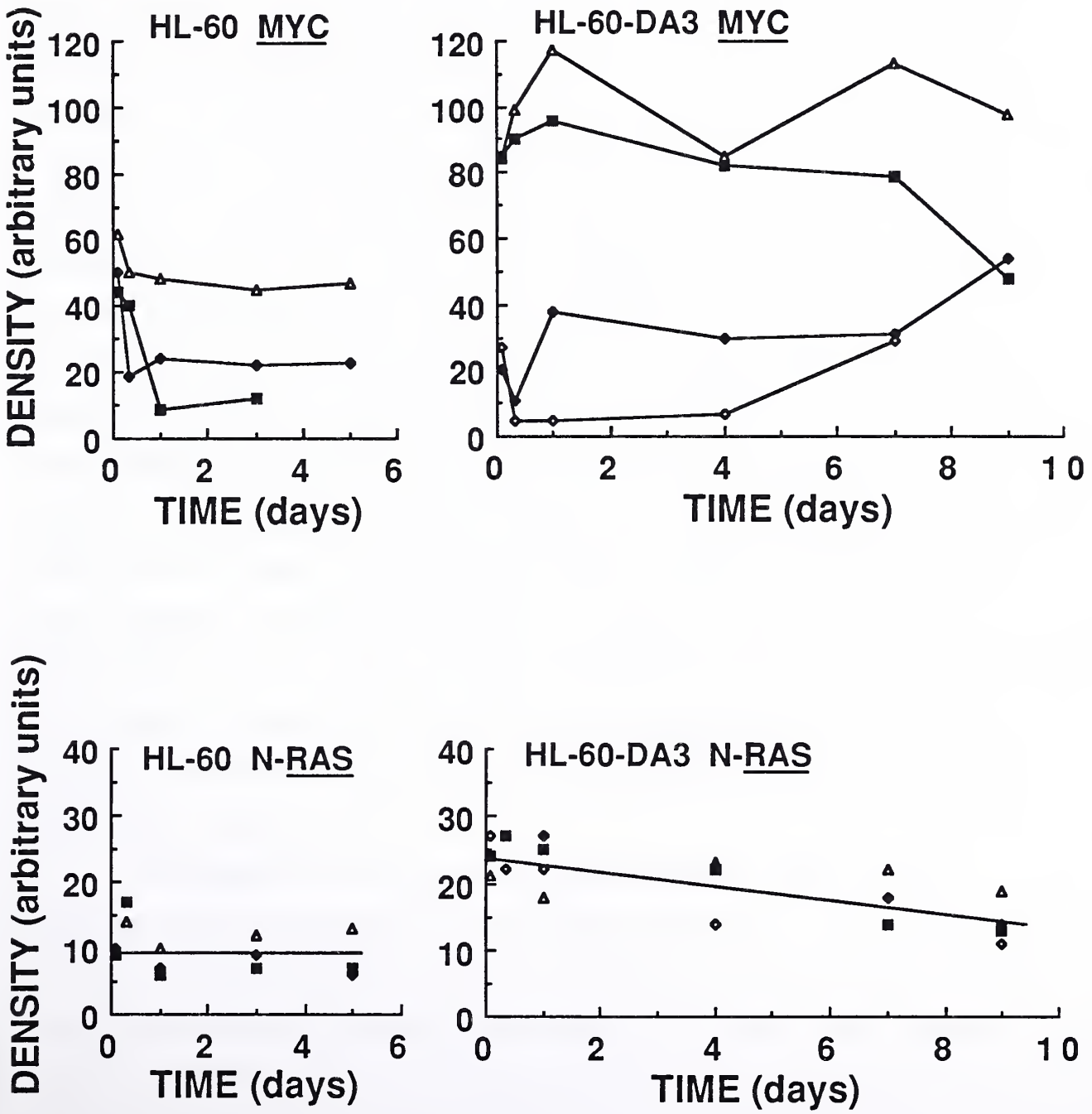


FIGURE 12: Densitometric measurements of cytodots represented in Figure 11. N-*ras* and *myc* density scales are only approximately equal.

MYC AND N-RAS RNA EXPRESSION

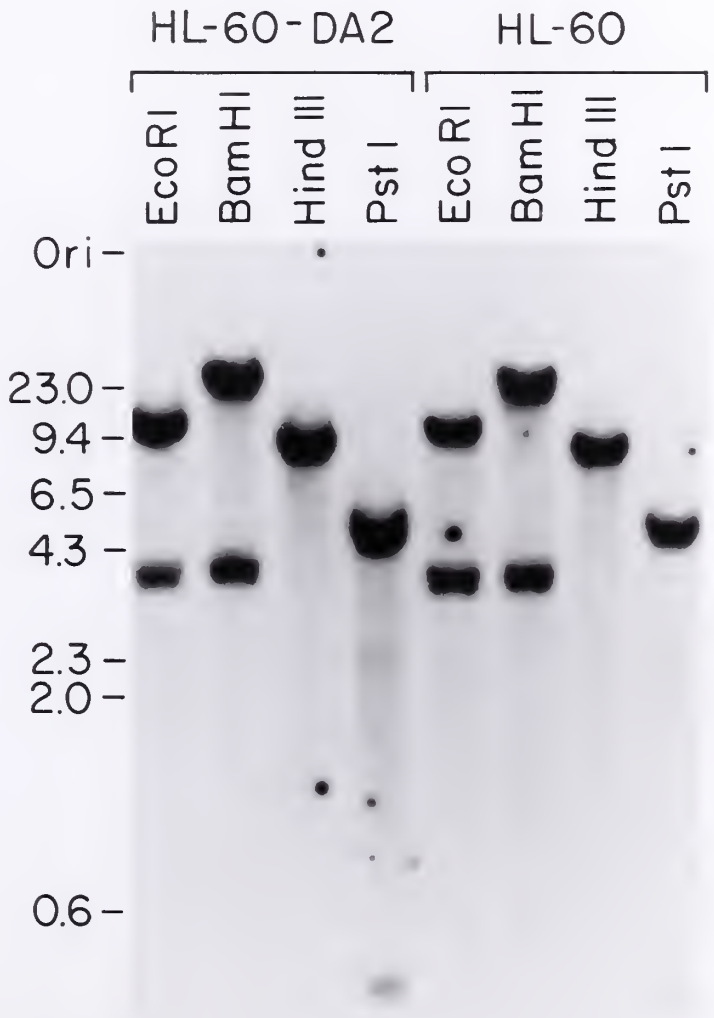


significantly in HL-60 cells induced to differentiate with either DMSO or RA. The decrease was evident as early as 2 hours and completed by 8 hours in the DMSO treated cells and by 24 hours in the RA treated ones. The untreated HL-60-DA3 cells showed myc RNA levels that were consistantly twice those of the untreated parental line. When treated with DMSO, which did not induce differentiation, a dramatic early decrease in myc expression occurred which returned to intermediate levels afte 1 day. The change in myc RNA expression in RA treated HL-60-DA3 cells, that also did not differentiate, was somewhat different than in the results with the HL-60-DA2 cells. Myc RNA levels stayed high for several days and then decreased to intermediate levels when the plateau phase was reached. When HL-60-DA3 cells were treated with both RA and DMSO, which induced only minimal differentiation, the changes in myc were similar to those noted with the HL-60-DA2 cells. Thus, an early dramatic drop occurred with a slight reaccumulation at the time of plateau growth. A similar reaccumulation at the plateau phase also occurred in the DMSO treated cells.

To analyze the myc genomic organization, DNA of the mutant line was analyzed by Southern blotting. Figure 13 shows no difference between the HL-60 and the HL-60-DA2 cells indicating that no gross rearrangement or significant change in the amplification of the myc gene occurred in the mutant line as compared to the parental line. All bands were in

FIGURE 13: Southern analysis of the myc genome in HL-60 and HL-60-DA2 cells. Twelve micrograms of total DNA were digested, separated and hybridized as described in Methods. Restriction enzymes and molecular weight markers are as indicated.

MYC GENE LOCUS IN HL-60 AND HL-60-DA2 CELLS



their previously reported locations (44,59,65,66), except for the 4.0 kB EcoR1 band and the high molecular weight band seen with BamHI digestion. The latter can be attributed to incomplete digestion (data not shown).

DISCUSSION

A DMSO Selected Differentiation Mutant Exhibits Cross Resistance to Retinoic Acid

An HL-60 mutant line that is resistant to DMSO and RA induced differentiation was developed and characterized. This line was then analysed for *myc* RNA expression in response to these agents. Although the line was created by selective pressure from constant DMSO exposure, it was found to also be resistant to RA induced differentiation, a finding previously reported for another DMSO resistant line (51). That the cells are indeed a differentiation mutant and not a mutant in the ability to respond to the NBT assay was shown by morphological analysis.

The changing characteristics of this line are noteworthy. First, when no selective pressure was maintained the line reverted back to the parental phenotype; whereas, when the selective pressure was continuously maintained the line became even more cross-resistant to the RA. This in itself is not too surprising in so far as parental cells usually have a growth advantage under the standard culture conditions and will thus eventually dominate the culture. The mutants with the greatest resistance, on the other hand, will dominate when selection pressure is maintained. It is not immediately clear whether the phenomenon is due to inducible genetic material in a clonal line (such as homogeneously

staining chromosomal regions or double minute chromosomes) or to a heterogeneous cell population. It has been shown that the HL-60 line has a very aberrant karyotype that does contain double minutes, homogeneously staining regions, and amplified genes (2,35,26,67,68). Genetic amplification that would then be lost in the abscence of selective pressure is thereby easily envisioned (69). However, given the large numbers of cells used and the previously reported karyotypic heterogeneity of the parental HL-60 cell line (2,67,68), it must be assumed that the mutant line is also a nonclonal and heterogeneous cell population.

With this heterogeneity and the lack of knowledge regarding the mechanism of differentiation produced by both DMSO and RA, it is difficult to dissect the exact mechanism by which the cells express resistance to differentiation. However, given the vastly different chemical structures and concentrations at which DMSO and RA are effective, it is not likely that their differentiation induction pathways are exactly the same. In fact, it has been postulated that RA exerts its effects through a specific RA binding protein (70,71), while DMSO may exert its effects through a nonspecific membrane interaction (72). In addition, the original isolate of the mutant line described here responded partially to RA and responded well to combined treatment, thereby giving further support to the possible existence of multiple differentiation mechanisms. Nevertheless, because the final

outcome of all differentiation inductions are very similar, the biochemical events produced by each of these mediators must eventually intersect. Thus, since the line described here eventually became resistant to both agents, the mutation almost certainly involves one of these intersecting events.

The Proliferating, Undifferentiated Phenotype Does Not Require Elevated myc Expression

Once the resistant line was established, I attempted to use it to probe for the role of the myc oncogene in differentiation. The presumed heterogeneous cell cultures and the changing phenotype made rigorous analysis of the measurements impossible. The changes in both myc and N-ras RNA expression in the parental line is well within the realms of previous reports (19,34). The changes seen with the cytodot technique, as mentioned before, are not quite as dramatic as in other reports but, once again, this was attributed to the high nonspecific signal seen with this technique. Also, the kinetics of the decrease in myc were somewhat more rapid than in a previously published report (19). The reasons for this are unclear, but careful reading and analysis of published HL-60 growth curves and response to standard differentiation inducers from different laboratories shows that significant variation occurs despite comparable culture conditions. Thus, the different kinetics of the decline in myc RNA may simply be an example of divergent cell line evolution.

The results with the myc oncogene in the mutant HL-60 line are at first glance confusing. However, some interesting conclusions can be made. The Northern gel of the HL-60-DA2 RNA clearly shows a marked decrease in myc RNA levels with both DMSO and RA treatment that is proportionally as great as that seen in the parental line when compared to the untreated control. Yet there is no or only limited differentiation. Thus, in this case, a decrease in myc RNA expression was not sufficient for differentiation to occur. Or looking at it in another way, high myc levels are not necessary to maintain the undifferentiated, proliferating, phenotype. It may be argued that it is not the relative amount of myc in a cell that is important but the absolute amount. The Northern blots leave this question open because myc RNA expression in both the RA and the DMSO treated cells, despite being relatively less than the untreated control, are still greater than the differentiating parental line. The myc RNA levels in the mutant cells treated with both RA and DMSO were decreased to the levels seen with DMSO treated parental cells, but they differentiate relatively well also.

The cytodot analysis, however, showed that HL-60-DA3 cells treated with both RA and DMSO have myc RNA levels that are at least as low as that seen in differentiating parental cells. In this case, though, they only differentiate to a limited extent. Thus, it seems that even an absolute decline in myc RNA levels is not sufficient to cause differentiation

in this line. The cytodot methodology also confirms the consistantly high constant myc RNA levels in the untreated mutant cells. In addition, the cytodot analysis corroberated the decline in myc RNA, without cellular differentiation, that occurs with DMSO treatment. The very early decline in myc with subsequent reaccumulation is reminiscent of the changes seen in differentiating Friend leukemia cells (39). One report also claims elevated myc expression at 24-48 h in differentiating HL-60 cells (73). Since it was not looked for, it is not known if a similar biphasic change occurred in the cells used for the Northern blots. The high myc RNA levels seen with RA treatment of the HL-60-DA3 cells as well as the rise in myc RNA levels seen in the DMSO and in the RA plus DMSO treated cells at the plateau phase of growth was not consistent with earlier results and is incompletely understood.

Finally, when attempting to define the importance of a putative mediator of a pathway, it is the function of the protein that is the physiologically important variable. Although myc protein and RNA levels have been correlated (74,80), until a good assay for myc protein function is developed, this limitation will remain. For now then, we will assume that a declining myc RNA level reflects a decline in the level and function of the myc protein.

In general myc RNA levels decline dramatically with HL-60 differentiation (19,34,41), as well as with the differen-

tiation of several other cell lines (38,40). Also, recent work shows that constitutive expression of myc inhibits differentiation in mouse erythroleukemia cells (44-46). Additionally, the kinetics of the decline in myc RNA parallels the kinetics of the decline in the clonability and NBT positivity of HL-60 cells but not the kinetics of thymidine incorporation into DNA upon the induction of differentiation (19). Thus, the data suggest that a decline in myc expression is necessary for differentiation.

However, there are many experimental systems in which increased myc RNA levels are related to the entrance of the cells into a proliferative state (74-83). Thus, it seems that increased myc expression is associated with the proliferative state, and in fact may be directly involved in DNA replication (84). Another way of looking at the decrease in myc levels seen with differentiation, therefore, is that it may simply be a reflection of the concomitant loss of proliferative capacity. Although elevated myc levels are insufficient to induce the replicative mode in some quiescent or differentiated cells (82), high myc levels could be sufficient to maintain the undifferentiated, proliferating phenotype (44-47). In this scenario, one could postulate that obligatory elevated myc expression occurs in all proliferating cells. Although this is certainly not true for all tumor cells (23,85), or even in all cells of a developing embryo (86), there has been little direct evidence for the

maintenance of the proliferating, undifferentiated phenotype in the face of declining levels of myc RNA.

This study is the first to describe such a pattern. That is, a decline in myc expression is not correlated with either the differentiated phenotype or the inhibition of growth in the HL-60 differentiation mutant described here. If the decline in myc is seen as mechanistically an important step in the maturation process, the obvious conclusion is that a decrease in the expression of myc is not sufficient for differentiation or, alternatively, the lesion in the differentiation pathway of this mutant is distal to the point at which a decline in myc RNA is initiated. On the other hand, as suggested above, myc expression can be seen as mechanistically important in maintaining the proliferating, undifferentiated phenotype. In that case, despite other studies which have shown that high myc RNA expression is sufficient for maintaining the undifferentiated, proliferating state (44-47), the conclusion of this study must be that it is not always necessary.

NOTE ADDED IN PROOF: Since writing this, Ely et al (Cancer Res. 47, 4595-4600) have described a HL-60 mutant partially resistant to phorbol dibutyrate induced monocytic differentiation in which myc expression initially declines dramatically but then reaccumalates at 15 to 30 hours postinduction.

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